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Tolerance Mechanisms of the Aromatic and Medicinal Plant *Salvia sclarea* to Excess Zinc

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Abstract: The responses of the aromatic and medicinal plant *Salvia sclarea* (clary sage) to 900 μM Zn exposure for 8 days in a hydroponic culture were investigated. The tolerance mechanisms under excess Zn exposure were assessed by changes in nutrient uptake, photosynthetic characteristics and leaf structure. The uptake and the distribution of Zn, as well as some essential nutrient elements such as: Ca, Mg, Fe, Mn and Cu, were examined by inductively coupled plasma mass spectrometry (ICP-MS). The results revealed that *Salvia sclarea* is a Zn accumulator plant that tolerates significantly high toxic levels of Zn in the leaves by increasing the leaf content of Fe, Ca and Mn ions to protect the photosynthetic function and even stimulate photosystem I (PSI) and photosystem II (PSII) activities. Additionally, the leaf photosynthetic pigments and the total phenolic and anthocyanin content were also studied. Data showed that the exposure to excess Zn significantly increases the synthesis of phenolic compounds in the leaves which plays an important role in the Zn detoxification and protection against oxidative stress. Lipid peroxidation and electrolyte leakage in leaves used as clear indicators for heavy metal damage were slightly increased. All these data highlight that *Salvia sclarea* is an economically interesting plant for phytoextraction and/or phytostabilization of Zn contaminated soils.

Keywords: Chlorophyll fluorescence; clary sage; nutrient uptake; oxidative stress; photosynthesis; phytoremediation; phytostabilization; photosynthetic pigments; phenolic content; Zn toxicity

1. Introduction

Toxic heavy metals lead to reduced plant growth, altered physiology and metabolism, as well as reduce plant cell integrity causing the generation of reactive oxygen species (ROS) [1,2]. They also interfere with the uptake of essential nutrients and water, and as a result crop yields decrease in heavy metal polluted soils [3]. Therefore, suitable plant species with a high capacity to accumulate heavy metals and an increased tolerance to their toxicity have been sought for the purposes of phytoremediation [2,4-7]. Plant metal accumulation varies within and between species (hyperaccumulators, accumulators and excluders), development stages, soil and metal type, time duration, etc. [3,4,8-11]. In agreement with the accumulation strategy proposed by Baker et al. [4], the hyperaccumulator species typically maintain high metal concentrations in their tissues without significant toxic symptoms, as the plants have established different mechanisms for detoxification and sequestration of heavy metals in nontoxic forms [1,2,11-15].

Zinc as micronutrient is one of the essential elements necessary for optimal growth, development and productivity of plants, since Zn is a co-factor of many enzymes, involved in the

biosynthesis of plant growth hormones, respiration and photosynthesis [16-18]. However, Zn ions in high concentrations exhibit phytotoxicity easily affecting the function of many enzymes and proteins, slowing plant metabolism and causing oxidative damage [18-21]. Visible Zn toxicity symptoms include reduced growth, leaf chlorosis (due to decreased chlorophyll content), necrosis, closure of stomata, disturbance in water balance [22-25]. The photosynthesis is considered the primary physiological process affected by heavy metals in all its phases directly or indirectly by ROS production damaging the photosynthetic apparatus of plants [23,26]. Heavy metals, including Zn, in higher concentrations also induce lipid peroxidation of the photosynthetic membranes, degrade photosynthetic pigments, inhibit the PSII activity and the electron transport chain, and decrease both carboxylation efficiency of Rubisco and net photosynthesis [21,26-28]. It has been also shown that Zn toxicity first affects chlorophyll content and then inhibits the photochemical activity of PSII [21]. In addition, the effects of Zn toxicity on the photosynthetic apparatus differ by the applied concentrations, the time of exposure, the plant species, etc. [11,23]. Chlorophyll fluorescence has been widely applied as a quick and a sensitive bio-indicator to measure the heavy metal stress on plants [15,28-35].

During the last years, there is a growing interest in aromatic plants (some herbs) that are considered suitable for environmentally safe phytoremediation, as their leaf essential oil is free of heavy metals [6,36-38]. The aromatic and medical plant clary sage (*Salvia sclarea* L.) is native to many Mediterranean countries and is one of the most important plants cultivated in the world as a source of essential oils for applications in human medicine or perfumery products [38-40]. This plant is also proposed to be a Zn and cadmium (Cd) accumulator, and a lead (Pb) hyperaccumulator with good potential for phytoremediation [38,41,42]. Favorable is also the fact that the heavy metals have almost no effect on the development of the clary sage grown on polluted soils, as well as this plant does not show signs of toxicity [38]. However, to the best of our knowledge, the Zn effects on the nutrient uptake and the function of photosynthetic apparatus, as well as the tolerance mechanisms of *S. sclarea* to high Zn concentrations have not yet been studied. Therefore, to explore some of the mechanisms involved in Zn tolerance of clary sage plants, this study was focused on the investigation of changes in the uptake and the distribution of essential nutrient elements (such as: Ca, Mg, Mn, Fe and Cu), as well as on some plant's defense mechanisms, that play an important role in the detoxification of high Zn levels, especially in leaves of *S. sclarea*, exposed to 900 μM Zn for 8 days in a hydroponic solution. The Zn tolerance was assessed also by measuring of the oxidative stress markers, changes in leaf photosynthetic pigments, polyphenolic and anthocyanin content, the leaf structure, as well as by studying the functional activity of the photosynthetic apparatus (PSII and PSI activities *in vivo*) using the chlorophyll fluorescence analysis and the P700 photooxidation.

Knowledge of these response mechanisms will be useful for the assessment of some tolerance strategies against Zn stress in this herbal plant, and to optimize management practices for phytoremediation.

2. Results

2.1. Zinc Accumulation and Mineral Element Uptake in Salvia sclarea

Exposure of Salvia sclarea for 8 days to 900 μM Zn resulted to strongly increased Zn accumulation in both roots and leaves. The Zn content in roots (40060 ± 1200 μg g^{-1} DW, Figure 1a) was much higher than that in the leaves (1759 ± 53 μg g^{-1} DW, Figure 2a). The increased Zn uptake was accompanied with significantly increased (about 4,4 times) accumulation of Fe and Cu in roots (Figure 1b), as well as increased accumulation of Fe (by 38%), Mn (by 85%) and Ca (by 22%) in leaves compared to control plants (Figure 2). Despite the significantly increased Cu accumulation in roots, its translocation to leaves decreased (Figure 3), as the leaf content of Cu was similar to the control (Figure 2b). Under excess Zn exposure it was also observed a decreased Mg content in roots by 44% (Figure 1a) and in leaves by 10% (Figure 2a), which is related with an increased translocation factor from roots to aboveground parts (Figure 3). In addition to Mg ions, an increased translocation was also detected for Ca and Mn ions (Figure 3). Translocation factor, showing a plant's ability to

translocate metal ions from its roots through stems to the leaves [43], was less than 1 for Zn, Fe and Cu ions and decreases under excess Zn (Figure 3).

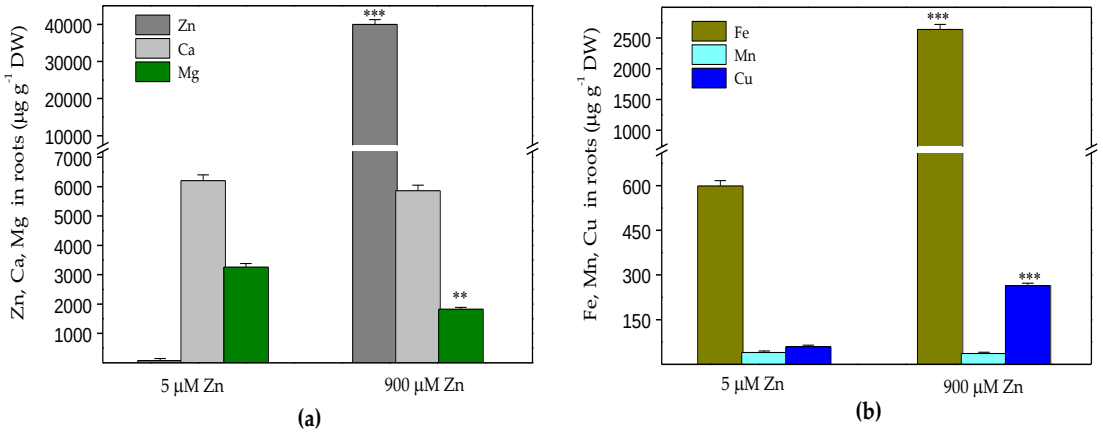


Figure 1. Content of Zn, Ca, and Mg **(a),** and Fe, Mn, and Cu **(b),** in *Salvia sclarea* roots expressed as µg g^{-1} dry weight (DW) after 8 days exposure at 5 (control) or 900 µM Zn. Mean values (±SE) were compared between the two Zn exposure for the same mineral element using Student's t-test and the differences were considered as statistically significant with **$p < 0.01$ or ***$p < 0.001$.

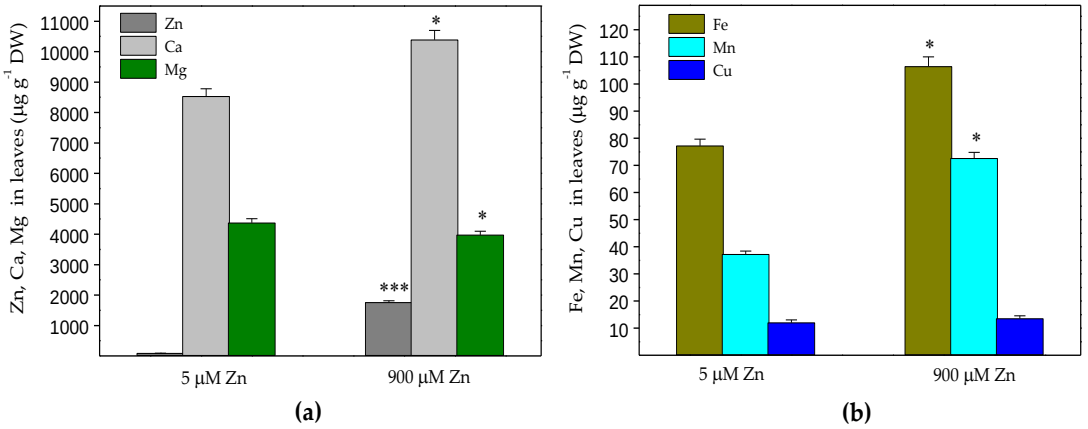


Figure 2. Content of Zn, Ca, and Mg **(a),** and Fe, Mn, and Cu **(b),** in *Salvia sclarea* leaves (µg g^{-1} DW) after 8 days exposure at 5 (control) or 900 µM Zn. Mean values (±SE) were compared between the two Zn exposure for the same mineral element using Student's t-test and the differences were considered as statistically significant with *$p < 0.05$ or ***$p < 0.001$.

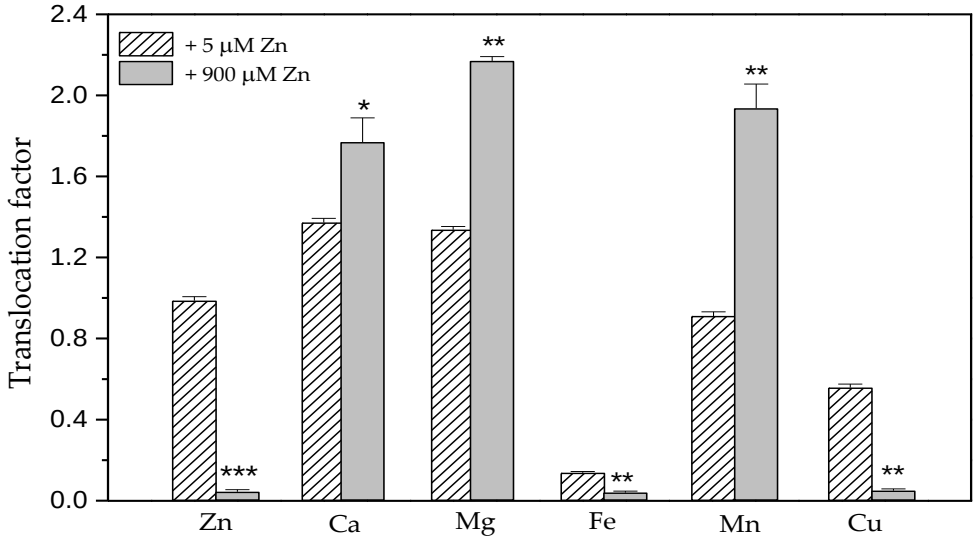


Figure 3. Changes in the translocation factor of the trace elements in *Salvia sclarea* plants in response to 5 µM (control) or 900 µM (excess) Zn exposure for 8 days. Mean values (±SE) were compared between the two Zn exposure for the same element using Student's t-test and the differences were considered as statistically significant with * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

2.2. Oxidative Stress Markers

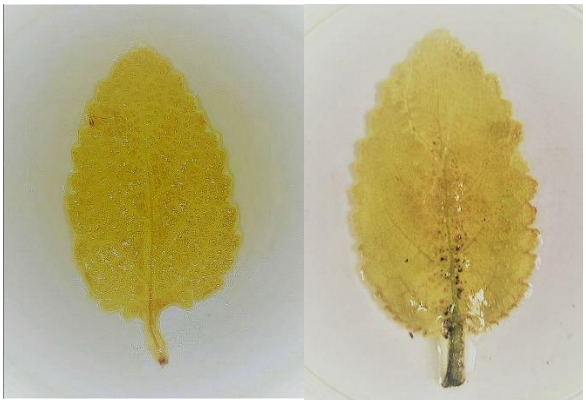
Following the changes in the relative water content (RWC) and electrolyte leakage (EL) of leaves of *S. sclarea* subjected to high Zn exposure the impact of Zn on leaf cell membrane integrity was evaluated. Results revealed that Zn treatment had a weak negative effect on leaf membrane permeability of clary sage plants and led to a slightly increased EL values as compared to the control leaves (Table 1) without exhibiting any other signs of toxicity (i.e., chlorosis, necrosis, rolling in leaves or disturbances in plant water-balance, Supplemental Figure 1S). Additionally, the oxidative stress and lipid peroxidation in clary sage leaves caused by the high-level Zn exposure were estimated by hydrogen peroxide (H_2O_2) and by malondialdehyde (MDA) contents as an indicator for membrane peroxidation levels. Data presented in Table 1 showed that these oxidative stress markers slightly increased by about 28% for H_2O_2 and 21% for MDA.

Histochemical detection of H_2O_2 overproduction in leaves of Zn-stressed *S. sclarea* plants indicated that the high Zn exposure caused accumulation of H_2O_2 mainly at the base of the leaf and around the midrib (Figure 4) without ongoing oxidative stress throughout the whole leaf.

Table 1. Effects of 5 µM (control) or 900 µM Zn exposure for 8 days on the relative water content (RWC), electrolyte leakage (EL), the content of H_2O_2 and MDA in leaves of *Salvia sclarea.*

	RWC (%)	**EL** (%)	**H_2O_2** (µmol g^{-1} FW)	**MDA** (µmol g^{-1} FW)
5 µM Zn	93 ± 2 ^{a}	9.9 ± 0.4 ^{b}	34.27 ± 1.26 ^{b}	25.97 ± 1.73 ^{b}
900 µM Zn	89 ± 2 ^{a}	12.1 ± 0.9 ^{a}	43.86 ± 2.18 ^{a}	31.54 ± 1.18 ^{a}

Different letters indicate significant differences between the means (±SE) for the same parameter ($p < 0.05$).



(a) **(b)**

Figure 4. Histochemically detected hydrogen peroxide (H_2O_2) in leaves of *Salvia sclarea* forming brown precipitates with 3,3'-diaminobenzidine (DAB) under **(a)** 5 μM Zn (control), and **(b)** excess Zn levels (900 μM).

2.3. Leaf Pigments and Total Phenolic Content

Measurement of leaf pigments was used as a sensitive biochemical marker for metal stress and phytotoxicity. After Zn treatment of *S. sclarea* plants for 8 days, the content of chlorophyll *a* (Chl *a*) slightly decreased by about 8%, while the content of Chl *b* and total carotenoids (Cars) did not change compared to those of the control leaves (Figure 5). Results revealed also that compared to the control clary sage plants, the leaf content of total phenolics and anthocyanins increased under the excess Zn exposure by 44% and 40%, respectively.

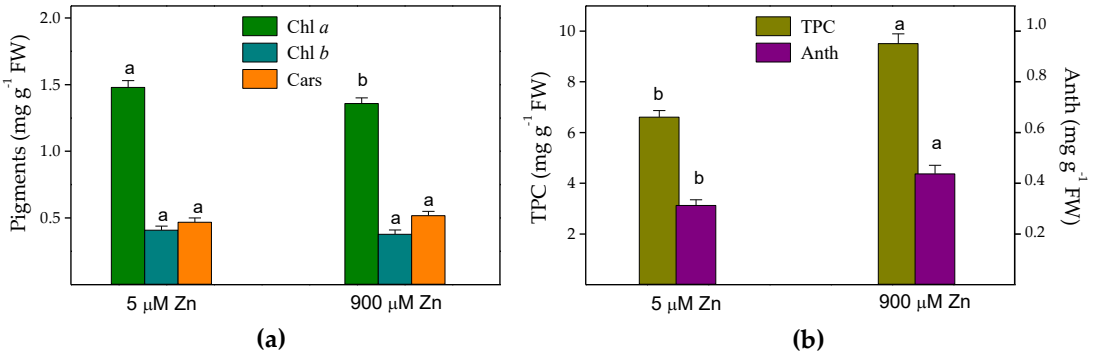


Figure 5. Effects of 5 μM (control) and 900 μM excess Zn exposure for 8 days on (**a**) the content of chlorophylls (Chl *a*, Chl *b*) and carotenoids (Cars), and (**b**) the total phenolic content (TPC, expressed as mg of gallic acid equivalent per g FW) and the anthocyanins (Anth, expressed as mg of cyanidin-3-glucoside equivalent per g FW) in leaves of *Salvia sclarea*. Different letters indicate significant differences between the mean values for the same parameter ($p < 0.05$).

2.4. Chlorophyll Fluorescence Analysis of Salvia sclarea Leaves under Zn Exposure

Here we estimated the maximum efficiency of PSII photochemistry (F*v*/F*m*), as well as the quantum efficiency of PSII photochemistry (Φ_{PSII}), the quantum yield of regulated energy dissipation in PSII (Φ_{NPQ}), the quantum yield of non-regulated energy dissipation in PSII (Φ_{NO}), and the fraction of open reaction centres (q*p*). After 8 days to excess Zn exposure (900 μM), the F*v*/F*m* did not differ

compared to the control (5 μM Zn), while Φ_{PSII} increased by about 13 % ($p < 0.01$) (Figure 6a). Due to this increased Φ_{PSII}, a significant decrease in Φ_{NO} and Φ_{NPQ} compared to controls, was detected (Figure 6b). These three quantum yields (Φ_{PSII}, Φ_{NPQ}, and Φ_{NO}) are equal to 1 assuming that the absorbed light energy is either utilized or dissipated. The increase of Φ_{PSII} compared to the control after the 8-days Zn exposure, was due to a significant ($p < 0.01$) increase in the fraction of open PSII reaction centres (q*p*), compared to the control. Under 900 μM Zn exposure a total of 89% reaction centres were open, while under control conditions (5 μM Zn) only 79%.

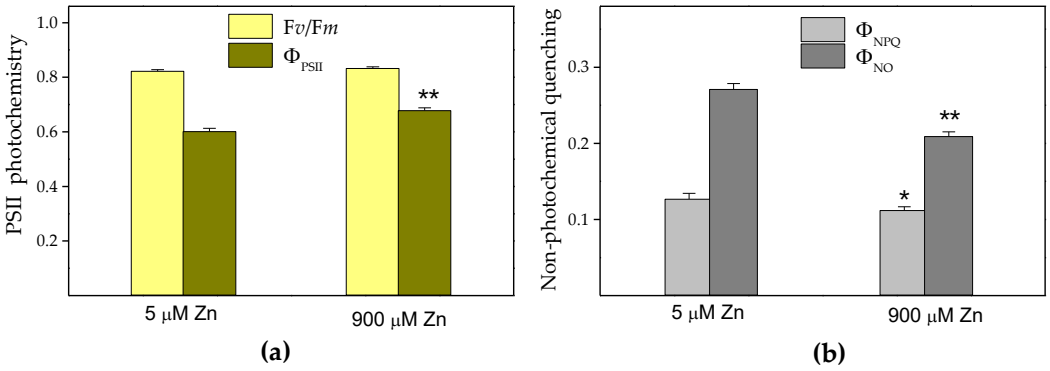


Figure 6. Changes in the maximum efficiency of PSII photochemistry (F*v*/F*m*) and the quantum efficiency of PSII photochemistry (Φ_{PSII}) **(a)**, as well as in the quantum yield of regulated energy dissipation in PSII (Φ_{NPQ}) and in the quantum yield of non-regulated energy dissipation in PSII (Φ_{NO}) **(b)**, of *Salvia sclarea* leaves after exposure to 5 μM (control) and 900 μM (excess) Zn for 8 days. Mean values (±SE) were compared between the two treatments for the same parameter using Student's t-test and the differences were considered as statistically significant with *$p < 0.05$ or **$p < 0.01$.

2.5. P700 Photooxidation

The measurements of the steady-state P700 photooxidation ($P700^+$) by far red (FR) light-induced absorbance changes at 830 nm (ΔA_{830}) were conducted to access changes in PSI photochemistry of *S. sclarea* leaves after high level of Zn exposure for 8 days. The $P700^+$ reduction after turning off the FR light is characterized by an exponential decay (with half-time, $t_{1/2}$) as shown in our previous study [44]. Results presented in Table 2 demonstrated that the amount of $P700^+$ (measured as $\Delta A/A_{830}$) was increased by 18% ($p < 0.05$) after the 900 μM Zn exposure in comparison to the control (5 μM Zn). Furthermore, the subsequent half-time ($t_{1/2}$) of $P700^+$ dark reduction was not statistically different from that in control plants.

Table 2. Effects of high level of Zn exposure on the FR light induced P700 photo-oxidation (P700+) and the kinetics of P700+ dark reduction (half-times, $t_{1/2}$) in leaves of *Salvia sclarea*. $\Delta A/A_{830}$ - the relative amplitudes of P700+ absorbance changes at 830 nm.

	$P700^+$ ($\Delta A/A_{830}$ x 10^{-3})	**$t_{1/2}$ (s)**
5 μM Zn	11.46 ±0.35 [b]	2.31 ± 0.22 [a]
900 μM Zn	13.52 ±0.43 [a]	1.97 ± 0.18 [a]

Different letters indicate significant differences between the mean values (±SE) for the same parameter ($p < 0.05$).

2.6. Leaf Anatomy under Zn Stress

Zink excess slightly affected *S. clarea* leaf anatomy (Figure 7), and the leaf basic structure remained unaltered (Figure 7b *cf* 7a). However more stomata could be observed (circles in 7b) and the epidermal cells contained darkly-stained material (arrowheads and asterisks in Figure 7b).

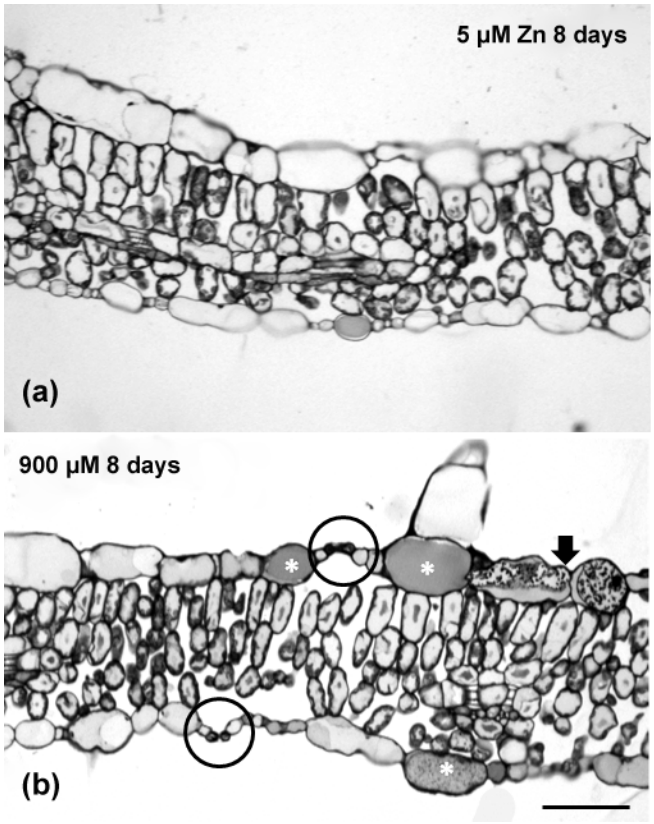


Figure 7. Transverse sections of *S. clarea* leaves, from (**a**) control and (**b**) 900 μm Zn 8 day-treated plants. Upon Zn excess application increase stomata could be observed (circles in **b**) while epidermal cells contained darkly-stained material (asterisks-arrows in **b**). Scale bar: 100 μm.

3. Discussion

Recently, it has been found that *S. sclarea* plants grown in heavy metal polluted areas, accumulated heavy metals through the root system and then translocated them to the aboveground parts [38,42]. Our data indicated more Zn accumulation in the roots than in the aboveground parts as the translocation factor decreased significantly (Figure 3). A similar decrease in the translocation of heavy metals to the aboveground parts was observed in sage plants (*Salvia officinalis*) grown on contaminated soils [45]. The decreased Zn translocation was also found to happen in some hyperaccumulating plants when grown hydroponically [17,34,46]. Moreover, the translocation factor less than 1 suggests that plants remediate Zn by phytoextraction through phytostabilization, concentrating the metals in the roots [43,47]. Therefore, when grown hydroponically, *S. sclarea* phytostabilises Zn in its roots.

Zn leaf value for hyperaccumulation is 10000 μg g^{-1} leaf DW (>1% DW) [4,9]. In our experimental conditions *S. sclarea* leaves do not reach these values, therefore, the clary sage can be referred to the Zn accumulators confirming previous observations with these plants grown on contaminated soils [38]. The presence of 8.0–100 μg Zn g^{-1} DW has been suggested to assist normal growth and development of plants, but the elevated content of 300 μg g^{-1} DW (>0.03% DW) was considered toxic for plants [18,48] and cause over production of ROS [21]. In this study after excess Zn exposure, we detected high Zn concentrations of about 1759 μg Zn g^{-1} DW (>0.17% DW) in the leaves (Figure 2a),

where the photosynthesis takes place, without exhibiting any symptoms of toxicity (i.e., chlorosis, necrosis, rolling in leaves or disturbances in plant water-balance) (Supplemental Figure 1S, Table 1) or affecting leaf structure (Figure 7). Similar high Zn concentrations were also measured in the leaves of the hyperaccumulator *Noccaea caerulescens* grown hydroponically upon 800 μM Zn supply [34].

It has been well established that Zn can interfere with the uptake of some other trace elements leading to an imbalance in nutrient uptake, transport and use [see in 25,34,49]. Previous study with hyperaccumulator *Noccaea caerulescens* under high Zn exposure has shown reduced uptake of Mn, Cu, Ca, and Mg ions, as well as enhanced uptake of Fe and Zn, while Ca and Mg concentrations in aboveground tissues remain unchanged and Cu increased significantly [34].

Our results demonstrated that the increased Zn uptake is accompanied with significantly increased accumulation of Fe and Cu ions in the roots (Figure 1b), as well as increased accumulation of Fe, Mn and Ca ions in leaves compared to the control plants (Figure 2). On the other hand, despite significantly increased Fe accumulation in roots and leaves (Figure 1b,2b), its translocation factor to aboveground tissues is decreased (Figure 3).

The observed increase of the nutrient elements in leaves of *S. sclarea* (for Fe, Ca and Mn) or the maintenance of almost the same (slightly diminished for Mg) concentrations as in the control plants is most likely included in the clary sage's protective strategy against Zn stress. Moreover, Fe, Ca and Mg cations have major roles in regulating (directly or indirectly) of the photosynthetic efficiency [49]. In contrast to previous reports [50], which found no effect or antagonistic effect of the Zn status on Fe uptake, here we observed a synergistic effect in Fe uptake that suggests a strategy of an increase in Fe accumulation as a response to a possible risk of Fe deficiency in leaves [reviewed in 25]. Iron is an essential trace element required for the respiration and the photosynthesis, and many fundamental biological redox reactions [25,51]. In the light reactions of photosynthesis in the leaves, it has been also found that Fe protects PSII from photoinhibition that occurs under Fe deficiency [51], as well as Fe supplement also maintaining the photosynthetic electron transport [52]. Therefore, the observed increased Fe accumulation in the leaves upon 900 μM Zn exposure could be one of the reasons for the increased quantum efficiency of PSII photochemistry (Φ_{PSII}) (Figure 6a), as well as the increased photooxidation of PSI (Table 2).

Excess Zn had also significant effect on the Mg ion uptake as the Mg content in roots decreased significantly (Figures 1a), however its translocation to aboveground parts strongly increased leading to slightly diminished Mg leaf content only by 10% (Figures 2a, 3). This was also accompanied with slightly decreased (by 8%) amount of Chl *a* in leaves, while no noticeable changes were detected in Chl *b* and Car content in the leaves (Figure 5a). Recently, a significant negative correlation was registered between the Zn concentration in the leaves and the amount of Chl *a* in *Trapa natans* L. recommending the leaf Chl *a* content as a sensitive biomarker for stress [53]. All above suggests for higher Zn tolerance of *S. sclarea*. At the same time, the Ca and Mn ion content in the leaves and their translocation factor in clary sage plants were enhanced under high Zn exposure (900 μM) in comparison to the low levels of Zn (5 μM) in nutrient solution (Figures 2, 3). It has been proposed that Ca cations are necessary not only for the normal function of the oxygen-evolving complex, but also for the regulation of Calvin cycle enzymes [54].

It is generally considered that extra ROS production under heavy metal stress is a key factor, which can promote lipid peroxidation of membranes causing disruption of their integrity (i.e. MDA and EL increased). Therefore, the contents of H_2O_2 and MDA are frequently used as index of oxidative stress. This study provided evidence that the excess Zn treatment induces alleviated oxidative stress since *S. sclarea* leaves displayed attenuated or no symptoms of toxicity coupled with lower H_2O_2 and MDA content (Table 1), and also less accumulation of H_2O_2 in whole leaves (Figure 4). In comparison to the control leaves, after excess Zn exposure the H_2O_2 content is higher with 28% leading to slightly enhanced (by 21%) lipid peroxidation (estimated as changes in MDA content) and EL values (Table 1), as well as no disturbances in the water balance (RWC) of the leaves was detected, suggesting for alleviated stress and higher tolerance of *S. sclarea* to Zn toxicity. Accordingly, the results observed by Jin et al. [55] have showed that elevated levels of Zn in the nutrient solution cause a significantly higher accumulation of H_2O_2 in the leaves of the non-hyperaccumulating ecotype of

Sedum alfredii leading to a strong increase (over 5 times) in MDA content, while in the hyperaccumulating ecotype this increase is very less pronounced up to 1000 μM Zn. A previous study with sage plants grown on heavy metal-polluted soil suggested that the neutralization of H_2O_2 is rather non-enzymatic than enzymatic process as registered weak activities of the most antioxidant enzymes [45]. In addition, the production of H_2O_2 not only can cause membrane damage, but it can also act as a long-distance signaling molecule activating antioxidant defence mechanisms in plants under stress [56,57].

It has been suggested that the increased leaf phenolic compounds in some herb plants have an important role in preventing oxidative stress thus increasing heavy metal tolerance of plants [58,59]. Therefore, the observed tolerance of clary sage leaves accumulating high Zn concentrations, most likely also due to the significantly increased amounts of total phenols and anthocyanins (Figure 5). In leaf epidermis the content of dark material (Figure 7), indicates probably an increase in phenolic content. Accordingly, recent study by Chen et al. [60] has found a reduced Chl *a* content accompanied by a significantly increased total phenolic content in leaves of *K. obovata* under high Zn concentrations, revealing that Zn plays major role in the synthesis, transport and metabolism of phenolic compounds, and thus enhances the heavy metal tolerance. Furthermore, Vidal et al. [61] have confirmed that plants which produce high amounts of phenolic compounds as a response to the heavy metal stress could be good candidates for phytoremediation and/or phytostabilization. Additionally, the anthocyanins have also been reported to have remarkably high antioxidant capacity, acting as ROS scavengers in vacuoles and thus counteracting against the toxic effects of heavy metals [1,62,63]. Therefore, their enhanced accumulation in clary sage leaves upon excess Zn exposure (Figure 5) probably acts as a barrier against heavy metal toxicity [64], as the formation of anthocyanin-chelate-metal complexes in plant tissue is also possible [65].

Generally, Zn excess was found to strongly affect leaf structure. More particular, Zn-treated poplar leaves increased in thickness with their pasalidae parenchyma to substantially increase in volume [66], while in Zn-treated *Hordeum vulgare* leaves a decrease in cellular size and intercellular spaces with an increase in metal concentration were recorded [67]. Zn-treated *S. sclarea* plants showed none of the above defects. Both control and excess Zn-treated leaves of *S.sclarea* had a single-layered epidermis on upper and lower surface of leaf (Figure 7). The bifacial leaves had a 2-3 layered palisade parenchyma and the spongy parenchyma consisted of irregularly shaped cells (Figure 7), having the typical anatomical features already reported by Özdemir and Şenel [68]. One other interesting feature was the increase in stomata number, which also found in peanut plants under Zn excess [69]. The increased stomata number enhance carbon uptake, while at the same time minimize water loss [70], and this could explain the non RWC disturbance observed (Table 1).

Since the photosynthetic efficiency is a sensitive bioindicator of environmental stress pressure [33], our data demonstrated stimulated PSII and PSI activity after the excess Zn exposure (Figure 6a and Table 2), while there were no changes in the dark-adapted F_v/F_m ratio (Figure 6a) or in the O_2 evolution (data not shown).

The analysis of the photooxidation of P700 to $P700^+$, reflecting the relative content of active PSI reaction centres, was used to assess the effects of high Zn accumulation in leaves on PSI activity *in vivo.* The level of $P700^+$ is suggested to be a direct and sensitive indicator of the electron acceptance capacity from PSI [71]. Our results revealed that the function of PSI in *S. sclarea* leaves was stimulated under excess Zn exposure for 8 days (Table 2). In contrast to the observed here tolerance of *S. sclarea* to high Zn exposure, our recent study [72] revealed that 100 μM Cd exposure for 8 days caused higher toxic effects in *S. sclarea* plants, expressed by a stronger reduction in the chlorophyll content, as well as by an inhibition of the oxygen evolution and the activities of both photosystems.

4. Materials and Methods

4.1. Plant Growth Conditions and Zn Treatment

Seeds of clary sage (Salvia sclarea L., the family Lamiaceae) were kindly provided by Bio Cultures Ltd. (Karlovo, Bulgaria) focused on herbs and essential oils production. After the initial germination, seeds were sown into pots filled with soil mixed with perlite (2:1 v/v) for about 6 weeks and then were transferred for 2 weeks into hydroponic containers (3-4 seedlings per container) filled with a continuously aerated nutrient solution (pH 6.0) described previously in details [35]. The seedlings were kept in greenhouse under 220 μmol m−2 s−1 photon flux density and 12 h light photoperiod at 25/20 °C. Uniform plants were selected and subjected to treatment with 5 μM (control) or 900 μM Zn (applied as $ZnSO_4$ and considered on the basis of earlier research [34]) in nutrient solution for 8 days. For each treatment were prepared 3 containers with four plants and the nutrient solutions were renewed every three days. Measurements were performed on the fully extended upper leaves of the plants.

4.2. Analyses of Zn and Nutrient Elemental Accumulation by Inductively Coupled Plasma Mass Spectrometry Method

After 8 days treatment with 5 μM (control) or 900 μM Zn, roots and leaves from treated plants were harvested, washed in deionized water and then dried at 75 °C to constant biomass, then milled and finally sieved. The dried tissue samples (~0.3g) were digested in close quartz vessels in a 3:1 ratio of 65% nitric acid and 30% hydrogen peroxide (Suprapur, Merck, Germany). The temperature of digestion was 200° using a microwave assisted digestion system Ethos One (Milestone Srl, Italy). Digested samples were quantitative transfer into polypropylene tubes and dilution with demineralized water (Direct-Q 3UV, Merc, German). In order to performed elemental analysis of Zn, Ca, Cu, Fe, Mg and Mn an Elan DRC II ICP-MS (PerkinElemer Sciex, Toronto, Canada) was used. Spectral interference has been eliminated using dynamic reaction cell (DRC) mode with high purity ammonia (Linde Gas, Poland) as reaction gas. The non-spectral interferences were reduced using a 10 ug L^{-1} solution of Ge and Rh as an internal standard. The series of standard solution for calibration was prepared by appropriate dilution of 10 mg L^{-1} multielement stock solution (Multi-Element Calibration Standard 3, Perkin Elmer Pure, MA, USA). Calibration curves were determined by the interpolation method. The analytical procedure was validated using the certified reference material NIST SRM Spinach Leaves 1575a. More detail information about ICP-MS operation condition, settings, and quality assurance are given in Appendix A.

4.3. Determination of the Oxidative Stress Markers

For the determination of electrolyte leakage (EL), some fully expanded leaves from different selected plants were cut into small pieces and placed in 40 ml tubes with distilled water for 24 h at 25 °C in the dark. After that the electrical conductivity of the solutions (EC1) was measured with a conductometer (Hydromat LM302, Germany), then the samples were boiled for 30 min and cooled to 25 °C and their final electrical conductivity was measured again (EC2). The electrolyte leakage (EL) was estimated from the equation: EL (%) = (EC1/EC2) × 100. The relative water content (RWC) of the leaves was calculated as described previously [73].

Fresh leaf samples (0.1 g) were immediately frozen in liquid nitrogen and stored at - 80°C for analysis of hydrogen peroxide (H_2O_2) and malondialdehyde (MDA) content. The determination of H_2O_2 content in leaves and levels of lipid peroxidation, by estimating MDA content were made as described by Mostofa et al. [74]. The histochemically detection of H_2O_2 in leaves by staining with 1% 3,3′-diaminobenzidine (DAB) solution were made following procedure described in [75]. The mean values (±SE) were averaged from three independent treatments with 3 repetitions for each treatment.

4.4. Analysis of Photosynthetic Pigments and Total Phenolic Content

Finely ground frozen leaf material (0.05 g) was extracted with an ice-cold 80 % (v/v) acetone. The homogenates were centrifuged at 5,000×g for 10 min at 0–4 °C, and the supernatant was measured spectrophotometrically (Specord 210 Plus, Ed. 2010, Analytik Jena AG, Germany). The amounts of chlorophyll *a* (Chl *a*), chlorophyll *b* (Chl *b*) and carotenoids (Cars), were calculated according to Lichtenthaler [76].

For estimation of the anthocyanin and total phenolic content, the frozen leaf samples (0.1 g) were extracted with 10 mL acidified methanol (1% HCl) in darkness at 0–4 °C for 2 day. The extracts were clarified by filtration and then used for analyses. Total phenolic content was determined by the Folin-Ciocalteu's colorimetric method as described in Sripakdee et al. [77]. The optical density was measured spectrophotometrically at 765 nm and phenolic content was expressed as mg of gallic acid equivalent (GAE) per g fresh weight (FW) of leaf tissues. Anthocyanins were estimated spectrophotometrically as the optical density of supernatant was measured at 535 and 657 nm and calculated as described in Mancinelli et al. [78]. Anthocyanin content was expressed as mg of cyanidin-3-glucoside equivalent per g FW. The mean values (±SE) were averaged from three independent treatments with 3 repetitions for each treatment.

4.5. Chlorophyll Fluorescence Analysis

Chlorophyll fluorescence analysis was performed using an Imaging *PAM M-Series* system (*Heinz Walz GmbH*, Effeltrich, Germany) as described in detail before [79]. Measurements were conducted in dark-adapted (20 min) *Salvia sclarea* plants grown with 5 μM or 900 μM (excess) Zn for 8 days. The chlorophyll fluorescence parameters that were calculated by the Imaging Win V2.41a software (*Heinz Walz GmbH*, Effeltrich, Germany) involved the maximum efficiency of PSII photochemistry (F*v*/F*m*), the effective quantum yield (Φ_{PSII}), the fraction of open reaction centers (q*p*), the quantum yield of regulated non-photochemical energy loss (Φ_{NPQ}), and the quantum yield of non-regulated energy (Φ_{NO}). The light intensity of 220 μmol photons m^{-2} s^{-1} was used for the photosynthetic efficiency measurements, similar to the growth light intensity.

4.6. Measurements of the Photooxidation of P700

The P700 photooxidation i.e., the oxidation of the PSI reaction centres (P700) to $P700^+$ [80], was measured by the far red (FR) light-induced absorbance transients at 830 nm (ΔA_{830}) using a PAM-101/103 fluorometer (Walz, Effeltrich, Germany) equipped with an ED-800T emitter-detector. The measurements were performed on dark-adapted leaves using FR light supplied by a photodiode (102-FR, Walz). The extend of FR-induced oxidation of P700 to $P700^+$ was estimated as the relative ratio $\Delta A/A_{830}$, where ΔA_{830} is the FR-induced absorbance change ($P700^+$) and A830 is the absorbance in darkness. The capacity of CEF was estimated by the half time of $P700^+$ dark reduction ($t_{1/2}$) signal after switching off the FR light as showed previously [44].

4.7. Light Microscopy

Pieces of *S. sclarea* leaves from plants exposed to 5 μM Zn (control) or 900 μM Zn for 8 days were prepared for chemical fixation as reported in [81]. Pieces were fixed firstly in a 3% paraformaldehyde + 3% glutaraldehyde solution buffered with 0.05 M sodium cacodylate pH 7.0 at room temperature for 6 h and subsequently, the leaf segments were post-fixed in 2% osmium tetroxide similarly buffered for 3 h. Afterwards they were dehydrated in an acetone series, treated with propylene oxide, and finally embedded in Durcupan ACM resin. An ultramicrotome (LKB 8801A, Stockholm, Sweden) equipped with a glass knife was used to obtained semi-thin sections (0.5–2μm) that were stained with 0.5% (w/v) toluidine blue O and observed with a Zeiss Axioplan light microscope equipped with a digital AxioCam MRc 5 camera.

4.8. Statistical Analysis

Average data are presented as the mean values (± SE) of three independent experiments with three repetitions each. Statistical analysis of means was performed using two-sampled Student's *t*-test. Differences were considered statistically significant at * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

5. Conclusions

To the best of our knowledge, this study revealed for the first time some of the tolerance mechanisms of the aromatic and medicinal plant *S. sclarea* to high Zn levels in the leaves, which include: (1) an altered uptake and distribution of some essential nutrients resulting in increased content of Fe, Ca and Mn ions in the leaves, and (2) an enhanced leaf content of non-enzymatic antioxidants, such as total phenolics and anthocyanins. Data presented here also suggest that these mechanisms are involved into the Zn detoxification and protection against the oxidative damage, thus protecting the photosynthetic performance and even stimulating PSI and PSII activities.

Future investigations should be performed to obtain more detailed information about metabolic pathways and enzymatic antioxidant mechanisms which could also contribute to enhanced Zn tolerance in *Salvia sclarea.*

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1,
Figure S1: Morphological comparison of *Salvia sclarea* plants exposed to 5 μM Zn (control) or 900 μM Zn for 8 days in a hydroponic solution.

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Conflicts of Interest: The authors declare no conflict of interest.

Appendix A

ICP-MS operation condition and setting

The study was carried out using an ICP-MS spectrometer equipped with a cyclonic spray chamber, concentric nebulizer of Meinhard type, quadrupole mas analyzer, and Pt cones. ICP-MS operation conditions were optimized daily. Those condition were: 1250W RF power; 16L min-1 the plasma gas flow rate; 0.89-0.91 L min-1 the nebulizer gas flow rate; 1.2 L min-1 the auxiliary gas flow rate. The daily performance of ICP-MS has been evaluated by the Smart Tune Solution, Elan DRC/Plus/II (PerkinElmer, MA, USA).

Quality assurance

In order to provide high quality results, the analytical procedure was validated. Parameters evaluated for the needs of the validation process included linearity, limit od detection (LOD), precision and trueness. The calibration curves were constructed daily and were based on the blank solution with analyte addition on six levels over a concentration range of: 0–1500 μg L−1 for 24Mg, 44Ca and 57Fe, 0–100 μg L −1 for 66Zn, 55Mn, 63Cu. The correlation coefficient R exceeded a value of 0.999 for all elements. The trueness of the analytical results was evaluated through the analysis of CRM and was expressed as recovery in percent (R,%). The percentages of recoveries for all elements varied from 95% to 106% respectively. The Student's t-test confirmed good agreement of trueness with certified values for all determined elements. Precision values, expressed as the coefficient of

variation in percent (CV,%) for all elements were as follows: from 1.6% to 3.7%. The limit of detection (LOD) was estimated based on the standard deviation of the 10 separate blank solution (2%HNO3) and slope of the curve (b), according the equation: LOD = 3.3S/b. The LODs estimated for the ICP-MS method were follows: 0.01µg g -1 (Zn), 30 µg g -1 (Ca), 0.05 µg g-1 (Cu), 40 µg g-1 (Fe), 0.8 µg g-1 (Mg) and 0.03 µg g-1 (Mn).

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